

## Phenotype Switching: Tumor Cell Plasticity as a Resistance Mechanism and Target for Therapy

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### Abstract

Mutations in *BRAF* are present in the majority of patients with melanoma, rendering these tumors sensitive to targeted therapy with BRAF and MEK inhibitors. Unfortunately, resistance almost invariably develops. Recently, a phenomenon called "phenotype switching" has been identified as an escape route. By switching from a proliferative to an invasive state, melanoma cells can acquire resistance to these targeted therapeutics. Interestingly, phenotype switching bears a striking resemblance to the epithelial-to-mesenchymal-like transition that has been described to occur in cancer stem cells in other tumor types. We propose that these changes are manifestations of one and the same underlying feature, namely a dynamic and reversible phenotypic tumor cell plasticity that renders a proportion of cells both more invasive and resistant to therapy. At the same time, the specific characteristics of these tumor cell populations offer potential for being explored as target for therapeutic intervention. *Cancer Res*; 74(21); 5937–41. ©2014 AACR.

### Introduction

Malignant melanoma is the most aggressive form of skin cancer, which is due to its propensity to rapidly metastasize. Whereas localized disease can be cured with surgery, treatment possibilities for metastatic melanoma have long been limited owing to minimal response of the disease to conventional chemotherapy. A decade ago, it was discovered that up to 70% of melanomas display genetic alterations in the MAPK pathway, with mutually exclusive mutations in *BRAF* (~50%) and *NRAS* (~20%) being the most prevalent (1). The most common mutation in *BRAF* results in a valine to glutamic acid substitution at codon 600 (*BRAF*<sup>V600E</sup>), rendering the BRAF kinase constitutively active.

The identification of hyperactivated MAPK signaling as a common driver of melanoma initiation and progression has led to the development of specific inhibitors targeting this pathway. Vemurafenib, a *BRAF*<sup>V600E</sup> inhibitor, was the first targeted melanoma drug to become standard of care in the clinic after demonstrating an impressive response rate (48%) and an increase in progression-free survival (5.3 months) compared with the chemotherapeutic agent dacarbazine (5% and 1.6 months, respectively), hitherto the standard-of-

care treatment (2). Initial excitement waned, however, once it became clear that despite this impressive initial response almost all patients developed resistance against BRAF inhibitors. Companion treatment, in the form of combining a *BRAF*<sup>V600E</sup> inhibitor (dabrafenib) with a MEK inhibitor (trametinib), resulted in more durable median response rates (3), but here too, resistance did eventually occur.

### Reactivation of the MAPK pathway confers resistance to targeted melanoma therapy

Multiple efforts have been undertaken to identify the molecular mechanisms underlying resistance to MAPK pathway inhibition (4, 5). These studies have revealed a strong dependency of melanomas on active MAPK signaling: around 70% of the uncovered resistance mechanisms to *BRAF*<sup>V600E</sup> inhibitors result in reactivation of this pathway. Examples include activating mutations in *NRAS*, as well as amplification or alternative splicing of the mutated *BRAF* gene (4, 5). Downstream reactivation of the MAPK pathway has also been described, for instance through activating mutations in *MEK1/2* (4, 5). Resistance toward the combination of BRAF and MEK inhibition also involves reactivation of the MAPK pathway, again through amplification or alternative splicing of *BRAF* or mutation of *MEK2* (6). Taken together, these large-scale genome-sequencing efforts underscore the importance of an intact MAPK pathway for melanoma survival.

Although other MAPK-independent mechanisms have also been identified, resistance still cannot be explained in up to 40% of all patient samples (4–6). This suggests that additional and perhaps alternative routes facilitating resistance, likely involving epigenetic events or stromal factors (7), remain to be discovered. New data, which will be discussed below, suggest that tumor cell plasticity, resulting in dynamic phenotypic heterogeneity, should not be ignored.

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### Phenotype switching as an alternative resistance mechanism

It was recently shown that melanoma cell lines with high levels of the transcription factor and master regulator of melanocyte development MITF (MITF<sup>high</sup>) were sensitive to either BRAF<sup>V600E</sup> or MEK inhibition, whereas MITF<sup>low</sup> cells displayed intrinsic resistance against these targeted drugs. Furthermore, expression of MITF inversely correlated with the expression of the receptor tyrosine kinase AXL (Mueller and Peeper, unpublished data; ref. 8). Interestingly, both *MITF* and *AXL* are part of a gene cluster that was identified almost a decade ago by RNA expression profiling of a large cohort of melanoma cell lines. These experiments revealed that cell lines could be clustered based on their metastatic potential, independent of their BRAF or NRAS mutation status (9). Cells with a so-called "proliferative" signature had a higher proliferation rate and expressed melanocyte differentiation markers, including MITF. In contrast, cell lines with an "invasive" signature displayed increased motility and migration, expressed genes involved in microenvironment modulation, had low levels of MITF, but higher levels of negative regulators of the Wnt/ $\beta$ -catenin pathway. This included Wnt5a, which had previously been implicated in melanoma metastasis (10, 11). Other studies have confirmed that melanoma cell lines segregate into these distinct expression clusters (12, 13).

The recent finding that MITF<sup>low</sup> cells are intrinsically resistant to targeted therapy (8) suggests that acquisition of an invasive phenotype results in resistance toward MAPK pathway inhibitors. Indeed, high expression of Wnt5a, a marker of the invasive phenotype, was found to induce resistance to BRAF inhibitors in patients (14). *In vitro*-acquired data show the same trend — the invasive phenotype can be linked to both intrinsic and acquired resistance to BRAF or MEK inhibitors (13, 15). Even more striking, proliferative melanoma cells can adopt invasive characteristics upon MAPK inhibition (16), indicating that targeted therapy may actually promote phenotype switching, potentially resulting in metastasis. This hypothesis is supported by the discovery that BRAF inhibition can induce invasion and metastasis *in vivo* when tumors are therapy resistant (17).

The effects of phenotype switching on therapeutic outcome should not be underestimated: whereas the proliferative population may be the major contributor to measurable tumor growth, the invasive cells might actually be the more problematic component. The above findings suggest that melanoma cells can use phenotype switching as a mechanism to acquire resistance, allowing them to evade growth arrest. As invasive cells seem less dependent on an intact MAPK pathway, reflected by their reduced sensitivity to BRAF- and MEK inhibition, phenotype switching may offer a crucial escape: it would essentially buy cells time to accumulate additional (epi) genetic alterations, which would then allow them to revert back to a proliferative phenotype and to survive in the presence of BRAF- or MEK inhibition.

### Epithelial-to-mesenchymal transition induces resistance to targeted therapy in other tumor types

Although phenotype switching was only recently (re)discovered in melanoma, which has its developmental origins in the

embryonic neural crest, the transition to an invasive state was already shown to induce resistance to targeted therapy in several epithelial tumors. For instance, an epithelial-to-mesenchymal transition (EMT) gene signature corresponds to resistance of non-small cell lung cancer (NSCLC) cell lines to the EGFR inhibitor erlotinib (18, 19). Resistant cell lines displayed mesenchymal features, including high vimentin and ZEB1 expression levels, and were more migratory, suggesting a switch to an invasive phenotype (20). Interestingly, these EMT features coincided with an upregulation of AXL, a marker of invasive cells in melanoma. Importantly, AXL was also upregulated in patients with NSCLC with acquired erlotinib resistance (21).

In other tumor types, including head and neck, bladder, and pancreatic cancer, resistance to EGFR-targeted therapies was also associated with EMT (22–24). Furthermore, an EMT switch was observed as resistance mechanism to other targeted therapies. For instance, HER2<sup>+</sup> breast cancer cells with high expression of the EMT markers Slug and Snail displayed intrinsic resistance toward the HER2-targeting antibody trastuzumab, which was reverted by knockdown of these transcription factors (25). Also, loss of MED12, which conferred resistance in several tumor types to multiple targeted therapies, including EGFR and MEK inhibitors, was shown to induce a partial EMT (26). Altogether, these data indicate that EMT is connected to therapy resistance. In fact, it suggests that tumors from different developmental origins can switch from a proliferative to an invasive phenotype to acquire resistance. The finding that the invasive gene signature previously determined in melanoma (9) contains genes that are involved in EMT further supports this hypothesis.

### Phenotype switching, EMT, and cancer stem cell plasticity: three names for the same phenomenon?

An intriguing finding is that invasive melanoma cells possess some features that are attributed to cancer stem cells (CSC) in other tumor types. CSCs are endowed with self-renewal capacities and are commonly held responsible for tumor initiation and maintenance, metastasis formation, and recurrence after (targeted) therapy. It was previously shown that immortalized mammary epithelial cells undergoing EMT acquire stem cell-like properties (27), suggesting a direct link between EMT and a "stemness" phenotype. This link is preserved in cancer, resulting in therapy-resistant tumor cells that harbor both invasive and self-renewal properties (28).

Importantly, CSCs from epithelial tumors appear to exist in two distinct states: a proliferative, epithelial state versus a migratory state that displays EMT features (29, 30). More importantly, these two populations are plastic and can interconvert (29, 30), thereby potentially playing a role in escaping therapy. For example, in HER2-amplified breast cancer cells, trastuzumab resistance resulted in expansion of the migratory CSC fraction (31). Strikingly, AXL overexpression, the above discussed marker of phenotype-switched and therapy-resistant melanoma cells, can induce EMT in breast cancer cells, whereas AXL inhibition reduces self-renewal and tumorigenicity of breast CSCs (32).

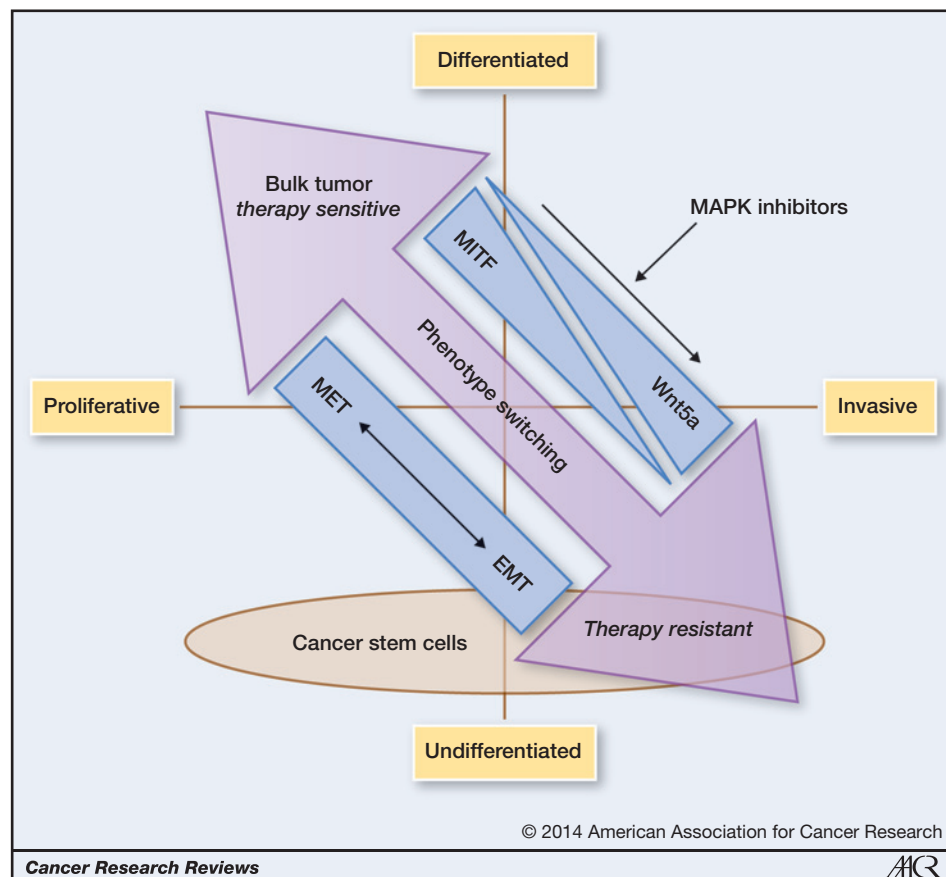
The shared features between epithelial CSCs undergoing EMT and (nonepithelial) melanoma cells undergoing phenotype switching are interesting, given that the existence of CSCs in melanoma is still debated. First, it has been shown that almost all melanoma cells, and not just a defined subpopulation, can initiate tumor growth and restore tumor heterogeneity upon transplantation into immune-deficient mice (33, 34). Second, although the H3K4 demethylase JARID1B was shown to be required for continuous growth and melanoma maintenance, its expression was dynamic and did not select for tumor-initiating cells (35), suggesting that the putative CSC population in melanoma is more plastic than the CSC population in other tumor types. Indeed, upon directly comparing melanoma cells with proliferative or invasive phenotypes, both populations were able to initiate tumor formation *in vivo*, although invasive melanoma cells required much longer to grow out than proliferative cells. Tumors initiated by cells with either a proliferative or an invasive phenotype were indistinguishable and showed heterogeneous MITF expression, suggesting that cells were able to switch their phenotype in both directions *in vivo* (9, 10). Thus, accumulating data support a model in which the bulk of melanoma cells have the potential to undergo phenotype switching. However, only a minority of the cells does so *in vivo* at any given time. In contrast, non-melanoma CSCs are generally shown to comprise only a minor fraction of all cancer cells, although it should be noted that

tumor cell plasticity remains incompletely understood: a large proportion of cancer cells may theoretically be able to acquire CSC properties *de novo* as a result of dedifferentiation.

Given that melanoma cell lines tend to retain their phenotype (whether proliferative or invasive) when cultured *in vitro*, phenotype switching may occur as a result of three-dimensional growth *in vivo*, perhaps in response to signals from the extracellular environment, similar to the way in which "stemness" can be thought of as a state that is determined by microenvironmental cues (e.g., a niche). Indeed, hypoxia signaling through HIF1 $\alpha$  was recently linked to both the induction of an invasive phenotype and therapy resistance in melanoma by inducing the expression of Ror2, a receptor for Wnt5a (15, 36). This confirms the previously suggested role for Wnt5a in melanoma cell invasion (10, 11, 37). Of note, a population of quiescent and migratory, mesenchymal-like CSCs was recently described at the invasive front of breast tumors, whereas a more proliferative, epithelial-like population of CSCs was located toward the center of the tumor, suggesting that this too could be regulated by the tumor microenvironment (30).

Taken together, the CSC and phenotype-switching hypotheses may be manifestations of the same underlying tumor cell plasticity, which might, at least partially, manifest itself as an EMT-like phenomenon (Fig. 1). Although the phenotype-switching model encompasses distinct phenotypes, which are

**Figure 1.** Tumor cell plasticity translates to a complex spectrum of phenotypic heterogeneity, which in turn affects the cells' sensitivity to drug treatment and propensity to metastasize. Both the phenotype-switching hypothesis and the CSC hypothesis support a continuous spectrum in which the cellular state is dynamic, rather than fixed. Tumor cells can switch phenotypes, such that they become more or less invasive/proliferative (as a result of EMT- and MET-like conversions) and more or less differentiated (according to their developmental history and extent of lineage specification). Switching can occur either as an intrinsic property of tumor growth in a three-dimensional environment or in response to therapy. Invasive, relatively undifferentiated melanoma cells (lower-right quadrant), which are more refractory to treatment, share many similarities with an EMT-like subset of CSCs. In contrast, current treatment strategies are aimed at eradicating the most proliferative cells, which may not necessarily be the most malignant.



either proliferative or invasive, a spectrum of phenotypes might exist in tumors, rather than two separate pools, much like is seen for EMT.

## Outlook

As more targeted therapeutics are finding their way into the clinic, the concepts discussed here are relevant for a broad variety of tumor types. Future strategies to identify novel drugs and active treatments will have to take into consideration that both proliferative and invasive (or CSC) populations should be targeted. This may require a change in how drug efficacy is assessed, such as the addition of secondary transplantation experiments in preclinical animal studies (38, 39). Given that melanoma cells can resort to phenotype switching as a therapy escape mechanism, it should be considered as a direct therapeutic target. The potential success is illustrated by a recent study (40), in which methotrexate was used to increase cellular levels of MITF, thereby forcing melanoma cells to adopt a proliferative, more differentiated phenotype associated with expression of the melanocyte-specific *TYR* gene. This in turn

sensitized the cells to a TYR-processed prodrug, which induced apoptosis in all melanoma cells (but not in nontransformed melanocytes), irrespective of their mutational status (40).

Summarizing, regardless whether phenotype switching is an active goal of the treatment itself, it is a phenomenon that cannot be ignored, especially given that some treatments might induce switching to the invasive phenotype, thereby promoting metastasis. A more in-depth understanding of tumor cell plasticity, as well as the challenges and promises it provides, will now have to make its way from bench to bedside.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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